APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1999, p. 404–408 Vol. 65, No. 2
0099-2240/99/$04.00+0

Composition and Enzymatic Activity of the Extracellular Matrix Secreted by Germlings of *Botrytis cinerea*†

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Received 20 July 1998/Accepted 30 October 1998

Germlings of *Botrytis cinerea*, an important fungal pathogen of plants, produce an extracellular matrix (ECM), or ensheathing film, that serves, in part, in their attachment (R. P. Doss, et al., Appl. Environ. Microbiol. 61: 260–265, 1995). The composition of this film has been ascertained by using samples obtained by growing germlings on a glass surface, removing the fungal mycelium by vigorous washing, and collecting the tightly attached film by scraping the substratum with a razor blade. Slightly over half of the dry weight of the ECM was found to be carbohydrates (about 20%), proteins (about 28%), and lipids (about 6%). Hydrolysis of the carbohydrate portion of the ECM revealed that glucose was the most prominent monosaccharide present, comprising about 60% of the total monosaccharides. Also present were mannose (about 35%) and *myo*-inositol (about 5%). The proteinaceous fraction of the ECM was made up of a number of polypeptides separable by polyacrylamide gel electrophoresis. The lipid fraction of the ECM, analyzed by thin-layer chromatography, was made up of several simple lipid components, including free fatty acid, mono- and triacylglycerol, wax ester, fatty alcohol, and several unidentified components. No complex lipids were detected. Isolated ECM exhibited polygalacturonase and laccase activity and was able to catalyze the hydrolysis of *p*-nitrophenyl butyrate, a model substrate for assessing cutinase activity. Cellulase, pectin lyase, and pectin methyl esterase activities were noted with both heated and unheated ECM preparations. Proteinase activity was not detected.

Botrytis cinerea Pers:Fr., a deuteromycete (Hyphomycete) fungus, is an important plant pathogen with an exceptionally broad host range (16). Conidia of *B. cinerea*, the primary inoculum source, first attach to substrata by a hydrophobic interaction that is easily disrupted and then, upon germination, attach strongly through secretion of an extracellular matrix (ECM) (7, 8). The ECM, also referred to as an ensheathing film or fungal sheath, is secreted by germ tubes and appressoria but not by the conidia themselves (8). The ECM is very resistant to removal from various substrata, brief exposure to a solution of strong base being the only known chemical treatment that can cause detachment of germlings (8).

The results described in this report were obtained from work undertaken to learn more about the chemical composition and enzymatic activity of the ECM secreted by germlings of *B. cinerea*. Assays for enzymatic activity focused on enzymes thought to be important in the infection process, defined by Nicholson and Kunoh (27) as "events that occur immediately after the pathogen comes into contact with the host and continue through the time of penetration." Mendgen et al. (24) have emphasized the importance of examining enzymes produced by the pathogen during penetration, rather than those secreted into a culture medium.

MATERIALS AND METHODS

Fungal culture. An isolate of *B. cinerea* (Bc-1) used in earlier studies was cultured on potato dextrose agar (Difco) as described previously (7, 8). Conidial suspensions were prepared in 0.1× potato dextrose broth (Difco).

Preparation of ECM. ECM preparations were obtained by inoculating 5- by 7.5-cm glass microscope slides with 1.5-ml aliquots of conidial suspension (about 10^6 conidia per ml). Slides with suspensions were incubated at 20°C and 100% relative humidity for 72 h. In most cases, sets of 18 slides were inoculated and incubated.

After incubation, the conidia and germ tubes were dislodged by directing a stream of water through a Pasteur pipette onto the surface of the slides (8). Washed slides were examined with a microscope equipped with interference contrast optics to make sure that no fungal tissue remained (Fig. 1) and stored in double-deionized water until several were accumulated. ECM was recovered by scraping the slides with a razor blade. Collected ECM and a small volume of accompanying water were then drawn from the edge of the razor blade into a plastic pipette tip by capillary action and transferred into a microcentrifuge tube. At no time during the collection process were the ECM preparations allowed to dry.

When ECM samples were to be used for enzyme assays, they were kept on ice during the entire collection process except during the washing step. Chemical composition was ascertained with ECM that had been dried in vacuo. In cases where analysis for lipids was to be carried out, the vacuum was broken with nitrogen gas, and dried ECM was stored at −20°C under nitrogen. All weighings of dried ECM preparations were done with a Cahn C-31 microbalance.

Estimation of ECM composition. Total carbohydrate in dried ECM was estimated by the phenol procedure of Dubois et al. (9) with a glucose standard. Protein was determined by the Lowry method (22) with a bovine serum albumin standard, and total lipid was estimated by measuring the weight loss of a proteinase-treated and washed ECM preparation after extraction with chloroform-methanol (2:1, vol/vol). Finally, an assay for total phenolics was carried out by the method of Singleton and Rossi (35) with gallic acid used as a standard.

Monosaccharides present in an acid hydrolysate of the carbohydrate portion of the ECM were identified by gas chromatography of alditol acetates (1) on a packed column of 3% SP2340 on 100/120-mesh Supelcoport. The ratios of lipid classes present in the chloroform-methanol extract of the ECM were determined by thin-layer chromatography (TLC) (4). A two-dimensional system was used initially to detect and separate the components in the total lipid extract. This system employed Whatman K6F silica gel plates with a first-dimension solvent system consisting of chloroform-methanol–7 M NH_4OH (65:30:4, vol/vol/vol) and a second-dimension solvent system consisting of chloroform-methanol-acetic acid-water (170:25:25:6, vol/vol/vol/vol). One-dimensional TLC was used to separate the simple lipid compounds. This system employed the same type of plate used for the two-dimensional separation, with a hexane-diethyl ether-formic acid (80:20:2, vol/vol/vol) solvent system. No effort was made to determine the molecular species present in the simple lipid classes detected. Proteins in the ECM were subjected to polyacrylamide gel electrophoresis (21) and detected by silver staining (45).

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† Technical paper 11429 of the Agricultural Experiment Station, Oregon State University.

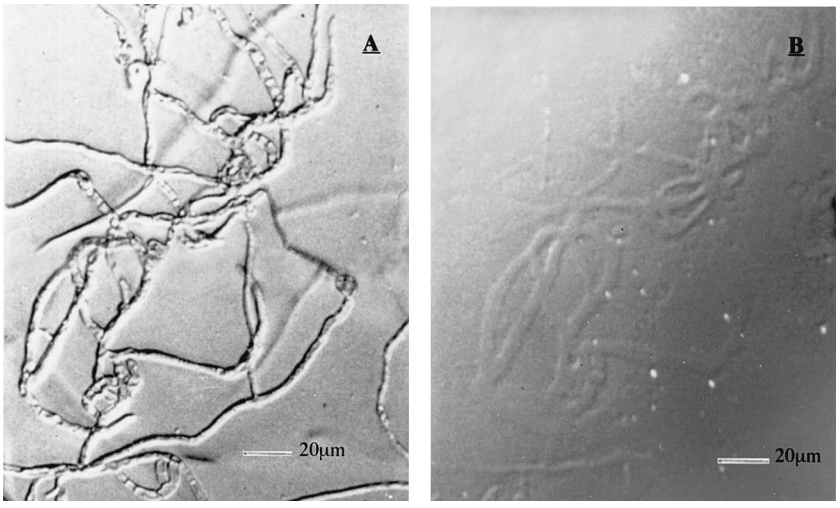

FIG. 1. Photographs made with a microscope equipped with interference-contrast optics of a glass slide on which conidia of *B. cinerea* were allowed to germinate and grow for 72 h (A) and the same slide after washing to remove the fungal mycelium (B). Note that the pattern of ECM left behind after washing mirrors that of the mycelium but that the mycelium is completely absent.

Enzyme assays. The activities of several enzymes that have been suggested to be involved in the infection process were examined by using modifications of published methods. Table 1 outlines some of the conditions of the assays. ECM was thoroughly dispersed in the assay solutions prior to the assay and, with the exception of the assay for cellulase, was collected by centrifugation, dried, and weighed after the assay. Polygalacturonase, pectin lyase, and cellulase were assayed by the cyanoacetamide method (14, 19). Ethanol-washed polygalacturonic acid (Sigma P3889) was used as a substrate for polygalacturonase, ethanol-washed pectin (Sigma P9561) was the substrate for pectin lyase, and water-washed cellulose powder (Sigma C1184) was the substrate for cellulase. In assaying cellulase, the reaction mixture was filtered (Millipore Ultrafree MC filter unit) to remove particulate cellulose prior to the addition of cyanoacetamide. For this assay the ECM dry weight was assumed to be 54 μg, the average weight of ECM obtained with the standard collection procedure.

Proteinase activity was evaluated by using azoalbumin as a substrate. After a solution containing azoalbumin had been exposed to an ECM preparation, the matrix was removed by filtration, and trichloroacetic acid was added. The amount of dye remaining in solution after the precipitation of the albumin and

TABLE 1. Enzyme activity possessed by ECM of *B. cinerea*

Enzyme (reference)	Buffer	Temp (°C)	Assay duration (h)	Approximate minimum activity detectable with commercial enzyme	Activity of ECM prep (range with two assays)
Cellulase (14)	0.05 M NaH_2PO_4–0.01 M citric acid (pH 6.3)–0.05% (wt/vol) cellulose powder–0.02% (wt/vol) sodium azide	30	3	3×10^{-3} U (1 U = 1 μmol of reducing groups as glucose released per min (cellulase; Sigma C1184)	1×10^{-6} to 3×10^{-6} U per μg of dry ECM with both heated and unheated samples
Esterase (6, 31, 34)	0.1 M Tris-HCl (pH 8)–3.7 mg of Triton X-100 per ml–0.42 mM *p*-nitrophenol butyrate–0.02% (wt/vol) sodium azide	25	1	No enzyme available; detection limit, about 10^{-5} μmol of *p*-nitrophenol released per min	1×10^{-7} to 4×10^{-7} μmol of *p*-nitrophenol released per μg of dry ECM per min; heat inactivated.
Laccase (36)	50 mM citric acid (pH 3.5)–2.5 mM 2,6-dimethoxyphenol	37	2		5×10^{-7} to 9×10^{-7} μmol of 3,3′,5,5′-tetramethoxy-diphenoquinone formed per min per μg of dry ECM; heat inactivated
Pectin lyase (14)	37.4 mM sodium acetate (pH 4.4)–0.2% (wt/vol) pectin–0.02% (wt/vol) sodium azide	30	3	10^{-6} U (1 U = 1 μmol of reducing groups as galacturonic acid released per min) (pectin lyase; Sigma P2804)	4×10^{-7} to 10×10^{-7} U per μg of dry ECM
Pectin methyl esterase (32)	0.45 mM KH_2PO_4 (pH 5.5)–0.4% (wt/vol) pectin–0.0015% (wt/vol) bromocresol green–0.02% (wt/vol) sodium azide	25	24	10^{-5} U (1 U = release of 1 μeq of protons as acetic acid equivalents per min) (pectinesterase; Sigma P1889)	10^{-5} U per μg of dry ECM with both heated and unheated samples
Polygalacturonase (14, 19)	37.4 mM sodium acetate (pH 4.4)–0.2% (wt/vol) polygalacturonic acid–0.02% (wt/vol) sodium azide	30	3	5×10^{-6} U (1 U = 1 μmol of reducing groups as galacturonic acid released per min) (polygalacturonase; Sigma P3429)	1×10^{-6} to 2×10^{-6} U per μg of dry ECM; heat inactivated.
Proteinase (38)	0.05 M KH_2PO_4 (pH 6)–25 mg of azoalbumin per ml–0.02% (wt/vol) sodium azide	30	1	10^{-4} U (1 U = release of color [A_{620}] equivalent to 1 mg of azoalbumin per min) (protease; Sigma P5147)	None detected

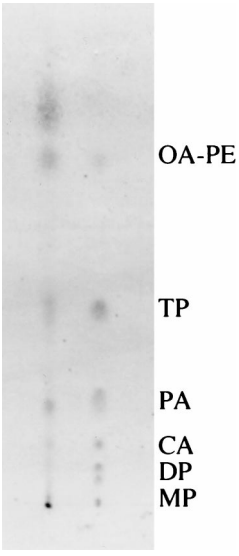

FIG. 2. Photograph of silica gel thin-layer plate showing several simple lipid components present in a lipid fraction (spotted on the left) from the ECM of *B. cinerea*. The plate was developed in hexane-diethyl ether-formic acid (80:20:2, vol/vol/vol), sprayed with methanol-H_2SO_4 (1:1, vol/vol), and heated on an aluminum sheet to bring about charring. Abbreviations for marker compounds (spotted on the right) are as follows: MP, monopalmitin; DP, dipalmitin; CA, cetyl alcohol; PA, palmitic acid; TP, tripalmitin; OA-PE, oleic acid-palmitolyl ester.

removal of the precipitate by centrifugation was taken as a measure of proteinase activity (38). Hydrolysis of *p*-nitrophenyl butyrate was assayed by spectrophotometric measurement of the release of *p*-nitrophenol (6, 31, 34). Pectin methyl esterase activity was evaluated by using absorbance changes of a solution of bromocresol green with pectin (Sigma P9561) as the substrate (32). Finally, to evaluate laccase activity of ECM preparations, the absorbance change caused by dimerization of 2,6-dimethoxyphenol was measured (36).

In every case, except when laccase activity was evaluated, sodium azide (0.02%, wt/vol) was included in the reaction buffer (azide is an inhibitor of laccase [36]). In addition, with each analysis a parallel reaction was run with a boiled ECM preparation. Commercial preparations of enzymes were used to test the assay procedures, except for laccase and esterase, for which no commercial preparations were available.

RESULTS

As noted in an earlier study (8), the ECM, which was formerly referred to as the fungal sheath, cannot be dislodged from glass slides even with vigorous washing. In contrast, fungal mycelium is readily removed by this treatment. In those cases where small traces of fungal mycelium remained on slides after washing, it could easily be seen by interference contrast optics (compare Fig. 1A and B) (8). Hence, the physical isolation of ECM from cytoplasm could be monitored visually, and complete separation could be assured (see Discussion).

Sets of 18 5- by 7.5-cm glass microscope slides inoculated with a conidial suspension of *B. cinerea* yielded, on average, 54.1 ± 2.0 μg of dried ECM (mean ± standard error [SE] for 20 preparations). Inspections of slides before and after washing suggested complete removal of fungal mycelium (Fig. 1). Several hundred micrograms of dried ECM, which was dark brown, was used for the chemical analyses.

The carbohydrate content of the ECM was estimated to be 20.2% ± 1.0% (mean ± SE for five determinations). Of the monosaccharides present in a hydrolysate prepared from the matrix, glucose was most abundant (about 60%), with lesser amounts of mannose (about 35%) and *myo*-inositol (about 5%) present. Arabinose, fucose, galactose, rhamnose, and ribose, as well as glucosamine and galactosamine, were not present at detectable levels.

Based on weight loss occurring as a result of the extraction of the ECM with chloroform-methanol (2:1, vol/vol), lipids were estimated to comprise 6.2% ± 1.1% (mean ± SE for four determinations) of the dry weight. When a total lipid extract of the ECM was subjected to two-dimensional TLC, a single spot characteristic in migration to simple lipids was observed (4). Among the simple lipid classes tentatively identified by one-dimensional TLC (4) were free fatty acid, mono- and triacylglycerol, fatty alcohol, and wax ester (Fig. 2). No complex lipids were detected. Migration of several components did not match the migration of any of the marker compounds.

Proteins were estimated to make up 28.1% ± 2.25% (mean ± SE for five determinations) of the dry weight of ECM. Polyacrylamide gel electrophoresis and silver staining (Fig. 3) revealed a number of protein components, with two prominent bands. The gels, which were run under denaturing conditions, were not subjected to activity staining.

Phenolic compounds were not detected in a 70-μg sample of ECM by a spectrophotometric method (35) in which 5 μg of gallic acid yielded an absorbance (at 765 nm) of 0.098. The test for phenolics was carried out because it was felt that the dark color of the dried preparation could be imparted by phenolic materials.

Taken together, carbohydrates, proteins, and lipids comprise about 54% of the ECM dry weight. The matrix appears to be quite hydrophilic, and it is possible that moisture taken up from the air accounts for the missing weight.

Proteinase activity could not be detected with ECM preparations, and cellulase and pectin methyl esterase activities were detected with both boiled and unheated preparations (Table 1). In two of three assays of pectin lyase, boiling failed to eliminate activity. In a third assay, a boiled sample of ECM was inactive. Polygalacturonase, laccase, and esterase (*p*-nitrophenyl butyrate-hydrolyzing) activities were readily demonstrated, and activity was greatly reduced or absent with boiled preparations. Laccase activity was not detected in the presence of sodium azide (36).

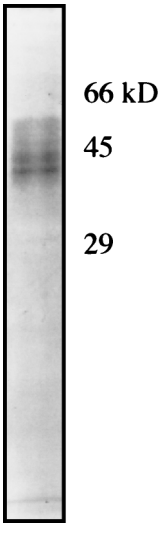

FIG. 3. Photograph of a polyacrylamide gel that was silver stained to reveal the proteins present in the ECM. The migration of molecular mass standards is shown.

DISCUSSION

The ECM secreted by germlings of *B. cinerea* is associated with the germ tubes and appressoria but does not ensheath conidia (8). A similar pattern of secretion has been observed with *Colletotrichum lindemuthianum* (28), another fungal pathogen of plants. It is not known how widespread this pattern of secretion of ECM is among germlings of plant pathogens, but, clearly, other patterns (e.g., secretion upon hydration of conidia [15]) do occur (24, 26).

As conidia of most *B. cinerea* isolates "are dependent on the presence of nutrients" (12) for germination (11), it is possible that ECM synthesis requires an exogenous supply of precursor compounds. Cole et al. (5) reported that an extensive matrix was formed by germlings of *B. cinerea* isolate P6g only when glucose was available to the germinating conidia. Without glucose, dry unwashed conidia formed only very short germ tubes with much less ensheathing material (5). Isolate P6g was anomalous in not requiring exogenous nutrients for germination (11, 12). With the strain of *B. cinerea* (Bc-1) used in this study, glucose alone was not sufficient to allow conidial germination. However, with a complex, albeit dilute, source of nutrients, such as 0.1× potato dextrose broth, nearly 100% germination was observed.

Glucose is the principal monosaccharide present in hydrolysate of the carbohydrate portion of the ECM. Dubourdieu et al. (10) characterized a β-(1-3)(1-6)-D-glucan that was secreted into the culture medium by *B. cinerea*. Pielken et al. (30) reported that a slightly modified form of the polymer, adherent to fungal mycelia, was also produced by the fungus. It is possible that this adherent polymer comprises the bulk of the carbohydrate fraction of the matrix. However, prolonged incubation in a solution containing Novozym SP-116, a complex of enzymes capable of catalyzing the hydrolysis of this glucan (30), did not cause detachment of fungal germlings from a glass substrate. (Novozym SP-116 was kindly provided by P. Stahmann.) Efforts to characterize the polysaccharide(s) present in the ECM are under way.

Also detected in the ECM were several simple lipid compounds. Complex lipids were not detected. Very little information is available on lipids of *Botrytis* spp., but, in general, the simple lipid classes present in the matrix have been reported to occur in various structures of other fungi (44). Complex lipids are universally present in eukaryotic cell membranes and thus can be considered markers for the presence of fungal cytoplasm. Therefore, their absence in ECM preparations verified the results inferred from microscopic examination: namely, that washing resulted in the isolation of ECM from fungal mycelium (see Results).

Kamoen (18) has suggested several possible roles for the ECM produced by *B. cinerea*. Among these are involvement in tropism toward the infection site, in preventing desiccation of germlings, and in providing a matrix in which fungal toxins or enzymes required for the infection process could be sequestered.

A number of enzymes are secreted into liquid culture by *B. cinerea*, and some of these are thought to be involved in infection (41). In particular, the cell wall-degrading enzymes, including endo- and exo-polygalacturonases, pectin lyase, pectin methyl esterase, and cellulase, have been studied. Polygalacturonase activity was readily detectable in ECM preparations and was eliminated by boiling. Pectin methyl esterase, pectin lyase, and cellulase activities were observed with both boiled and unheated preparations. In other studies it was reported that a cellulase from *B. cinerea* retained activity after boiling (39) and that pectin methyl esterase exhibited activity at 80°C (32). The response of pectin lyase to heating has not been reported.

Another enzyme thought by some to be important in the infection process is cutinase (20). (However, see reference 40.) Ungerminated conidia (13) of, and culture filtrate (34) from, *B. cinerea* possess cutinase activity, and modifications of the cuticle in the vicinity of the infection hypha (33) and at the site of penetration (23) have been noted. Cutinase activity can best be assayed by examining the hydrolysis of titrated cutin (20). However, because this substrate is somewhat inconvenient to prepare and use, many studies of cutinase activity use *p*-nitrophenyl esters of carboxylic acids, whose hydrolysis can be measured spectrophotometrically, as model substrates (20, 31, 34). ECM preparations from *B. cinerea* were able to hydrolyze *p*-nitrophenyl butyrate, suggesting that a cutinase could have been present.

In contrast to the cell wall-degrading enzymes and cutinase, which could be involved in breaching the physical barriers to entry into the plant, the lignin-degrading enzyme, laccase, is thought by some to play a role in protecting *B. cinerea* from plant defense compounds (2, 29). Suppression of the synthesis of laccase could increase the virulence of the pathogen toward some hosts (2, 3), and Bar-Nun et al. (2) stated that laccase is "a necessary but insufficient requirement for infection" by *B. cinerea*. Relatively large amounts of laccase were secreted by the fungus into the culture medium (36), and it was also readily demonstrated in ECM by using 2,6-dimethoxyphenol as a model substrate (36).

The ECM of *B. cinerea* is almost certainly involved in adhesion of germlings to the host plant surface (7). Moreover, several of the enzymes thought to play a role in the infection process are, as suggested by Kamoen (18), present in the matrix. The nature of the association of the enzymes with the matrix is not known; however, it is noteworthy that detectable levels of enzyme activity remain after ECM preparation, a process that involves several steps where diffusion into water could occur.

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